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Transforming Growth Factor β1 Positively Regulates Its Own Expression in Normal and Transformed Cells*

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Transforming growth factor β1 (TGF-β1) regulates the growth, differentiation, or function of nearly all cell types. We now report that TGF-β1 increases steady-state levels of its own message in six different normal and transformed cells in culture. Accumulation of TGF-β1 mRNA can be detected by Northern blot analysis within 3 h of addition of the peptide to cells, and enhanced message levels persist as long as TGF-β1 is present in the culture medium. This autoinduction is half-maximal at ~10 pM TGF-β1, and maximal stimulation corresponds to a 2–3-fold increase in transcript levels. In normal rat kidney cells, the rise in TGF-β1 mRNA is actinomycin D-sensitive and is accompanied by a parallel (~3-fold) increase in secretion of TGF-β1 protein in the culture medium of treated cells, as detected by immunoprecipitation of biosynthetically labeled ^{35}S-labeled TGF-β1 using specific anti-TGF-β1 antibodies. Treatment of normal rat kidney cells with either epidermal growth factor or platelet-derived growth factor also results in an increase in TGF-β1 mRNA (2–3-fold), although epidermal growth factor and TGF-β1 appear to act via distinct mechanisms since their combined effects are greater than additive.

Transforming growth factor (TGF)[1] β1 is a 25,000-dalton homodimeric molecule which belongs to a recently established family of structurally homologous proteins. Members of the TGF-β gene family exert variable effects on growth, differentiation, and function of a number of different cell types (for recent reviews, see Refs. 1–3). The relative abundance of TGF-β1 in platelets (4) and bone (5) as well as its specific association with certain tissues of the developing embryo (6, 7) indicate that TGF-β1 may play a role in wound repair processes in the adult and morphogenesis during gestation. Although TGF-β1 has been shown to exert a wide variety of effects (both stimulatory and inhibitory) on cell growth parameters and differentiated phenotypes, its molecular mechanism of action is not clear. In systems that have been examined to date and in which TGF-β1 has an inhibitory effect on cell growth, TGF-β1 does not stimulate the well-characterized biochemical signaling events associated with cellular activation, such as ion fluxes, protein kinase C activation, c-myc induction, or S6 protein phosphorylation, nor does it inhibit the activation of these events by mitogenic factors (8, 9). In fact, the earliest known effects of TGF-β1 on target cells involve modulation of gene expression. Indeed, TGF-β1 has been shown to increase expression of a number of matrix-associated and structural genes such as those for various types of collagen (10, 11), fibronectin (10), the fibronectin receptor (12), and actin (13), as well as genes encoding protease inhibitors such as TIMP (metalloproteinase inhibitor) (14) and plasminogen activator inhibitor 1 (15). TGF-β1 also enhances expression of growth regulatory molecules including c-*sis* (16), platelet-derived growth factor (PDGF) A chain (17), and interleukin-1 (18). Inhibitory effects of TGF-β1 on gene expression have also been observed including the genes for transin/stromelysin and collagenase (14, 19).

Although the list of genes regulated by TGF-β1 is rapidly expanding, little is known about those factors which regulate the TGF-β1 gene itself. It is known that TGF-β1 appears to be developmentally regulated (7) and that transformation by Harvey and Moloney sarcoma viruses is accompanied by increased levels of both TGF-β1 mRNA expression (20) and secretion of the protein (21). In addition, activation of both B (22) and T (23) lymphocytes results in a significant increase in TGF-β1 mRNA expression. We now report that increased TGF-β1 mRNA expression appears to be a general response to TGF-β1 treatment in several normal and transformed cell lines.

MATERIALS AND METHODS

Cells and Culture Conditions—Cells used in this study were grown in a humidified incubator (5% CO_2, 95% air) at 37 °C in the presence of antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin). Rat osteosarcoma cells (ROS 17/2) were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium containing 5% fetal calf serum. All other lines were cultured in Dulbecco's modified Eagle's medium containing either 5% calf serum for NRK-49F normal rat kindey fibroblasts and mouse NIH 3T3 cells or 10% fetal calf serum for the human cells including A549 (lung adenocarcinoma), HT1080 (fibrosarcoma), and MRC5 (normal lung fibroblasts).

Growth Factors and Reagents—TGF-β1 and epidermal growth factor (EGF) were purified to homogeneity from human platelets (4) and mouse submaxillary glands (24), respectively. Porcine platelet-derived TGF-β2 (25) was obtained from R & D Systems (Minneapolis, MN), and human PDGF was a gift of Dr. Thomas Devel (Washington University, St. Louis, MO). Cycloheximide and actinomycin D were purchased from Sigma; all reagents were of the highest grade commercially available.

RNA Extraction and Northern Analysis—Total RNA was isolated from cells following experimental treatment according to the guanidine isothiocyanate/cesium chloride procedure described by Chirgwin *et al.* (26). For Northern blot analyses, equal amounts of RNA (10–15 μg) were electrophoresed on 1% agarose gels containing 0.66 M formaldehyde and transferred to Gene Screen (Du Pont-New England Nuclear). Ethidium bromide (300 μg/ml) was included in both gel

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[1] The abbreviations are used: TGF, transforming growth factor; NRK, normal rat kidney; ROS, rat osteosarcoma; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; SDS, sodium dodecyl sulfate.

EXHIBIT A

and running buffers in order to visualize the positions of molecular weight standards (RNA ladder, Bethesda Research Laboratories) and ribosomal RNA by UV illumination following electrophoresis. Blots were hybridized using ^{32}P-labeled probes according to the method of Church and Gilbert (27).

Hybridization Probes—Labeling of the 218-base pair single-stranded TGF-β1 probe, complementary to the mature coding region of human TGF-β1 mRNA, has been described (28). The plasmid pRGAPDH-13 (29) containing rat glyceraldehyde-3-phosphate dehydrogenase cDNA and an 850-base pair fragment from the mouse α2(I) collagen plasmid pAZ1002 (11) were radiolabeled by nick translation.

Immunoprecipitation of Biosynthetically Labeled TGF-β1—Cells were grown to near confluence in 60-mm dishes and then shifted to medium containing 0.25 mCi/dish [^{35}S]cysteine (Amersham Corp.), 2% plasma-derived serum, and 10% of the usual concentration of methionine and cysteine. TGF-β1 was present at 5 ng/ml where indicated. After 20 h, the culture medium was harvested, and secreted TGF-β1 was immunoprecipitated as previously described (30). Equal amounts of radioactivity from trichloroacetic acid-precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli (31) on 10% acrylamide gels. ^{125}I-Labeled TGF-β was applied to gels as a control, and labeled proteins were revealed by autoradiography.

RESULTS

Auto-stimulation of TGF-β1 mRNA in Various Cultured Cells—We have examined the effect of TGF-β1 on expression of its own mRNA in a number of normal and transformed cells in culture. As shown by Northern blot analysis in Fig. 1 (*upper*), a single band representing the ~2.4-kilobase pair TGF-β1 transcript (32) is constitutively expressed in both normal (MRC5) and transformed (A549, HT1080, ROS) cells derived from different species and tissues in the absence of exogeneous factor (*lanes 1, 3, 5,* and *7*). However, when TGF-β1 is added to the serum-free culture medium of cells (*lanes 2, 4, 6,* and *8*), an increase in steady-state message levels for TGF-β1 is observed in all cell types. For Northern blot analyses, a strand-specific probe complementary to the 3′ end of TGF-β1 mRNA (28) was used to detect only sense transcripts. Variation in the quantity of RNA loaded on gels was controlled by monitoring expression of mRNA corresponding to glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in the glycolytic pathway that is not affected by TGF-β1 treatment. Quantification of the results shown in Fig. 1 (*upper*) shows that following 18 h of TGF-β1 treatment, TGF-β1 mRNA levels are 2–3-fold higher than untreated control levels (Fig. 1, *lower*). Normalized values for the stimulation of TGF-β1 mRNA levels, using glyceraldehyde-3-phosphate dehydrogenase as control, are 3-fold for A549 cells, 3.9-fold for MRC5 fibroblasts, 1.5-fold for HT1080 cells, and 2.3-fold for ROS cells.

Kinetics of the effect of TGF-β1 on accumulation of its own message in ROS cells are shown in Fig. 2. An increase can be detected within 3 h following TGF-β1 addition; it reaches a peak by 6 h and persists as long as TGF-β1 is present in the cell culture medium (>18 h). In contrast, steady-state message levels for glyceraldehyde-3-phosphate dehydrogenase in ROS cells did not vary as a function of time or treatment with TGF-β.

Autostimulation of TGF-β1 Expression in NIH 3T3 and NRK Cells—In an effort to learn more about the mechanism of this autoregulatory effect of TGF-β1 on mRNA levels, we focused our attention on two nontransformed cell lines, NIH 3T3 and NRK cells. The latter cell line has been the subject of numerous studies involving the effects of TGF-β1 on anchorage-dependent and -independent proliferation of cells (33–35) and on the enhancement of extracellular matrix formation (10, 11, 36, 37). In NIH 3T3 cells, the time course of TGF-β1's stimulatory effect on its own message levels is quite

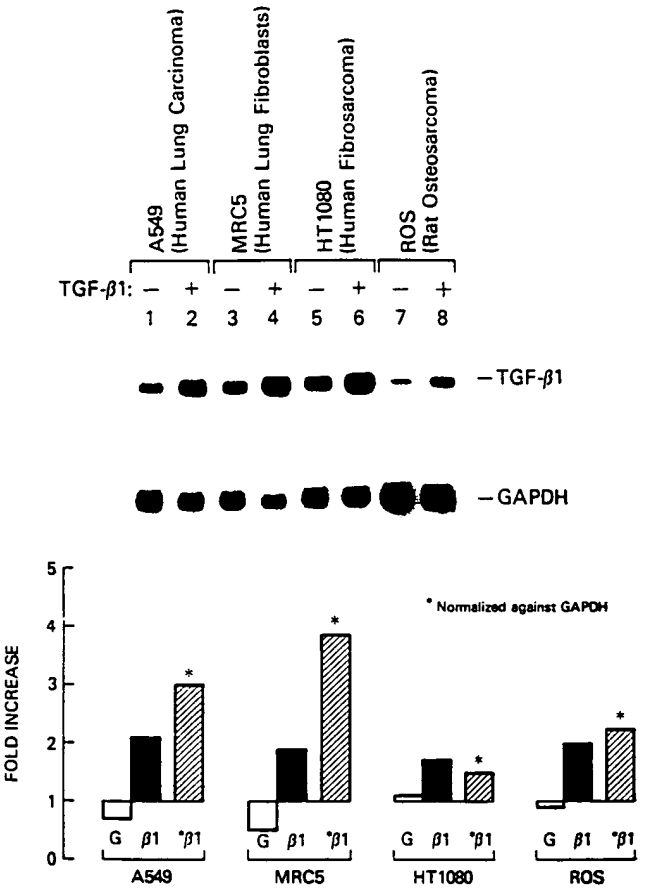

FIG. 1. Autoreguation of TGF-β expression in cultured cells. *Upper,* Northern blot analysis of total RNA isolated from cells following an 18-h incubation under serum-free conditions in the absence (–) or presence (+) of 5 ng/ml TGF-β1. The blot was hybridized using TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes; transcript lengths are 2.4 and 1.4 kilobase pairs, respectively. *Lower,* quantitative analysis of TGF-β1 effects on levels of mRNA for TGF-β1 (*closed bars*) and glyceraldehyde-3-phosphate dehydrogenase (*G*; *open bars*) by densitometric scanning of the autoradiograms (*upper*); values for mRNA levels of untreated cells have been set equal to 1, and those from TGF-β-treated cells are expressed as -fold stimulation. -Fold increase, normalized against glyceraldehyde-3-phosphate dehydrogenase mRNA levels, is represented by *hatched bars* (*).

similar to that seen in ROS cells (Fig. 3, *upper* and *lower*), that is, an effect is manifest by 3 h of treatment and maintained in presence of TGF-β1. In NRK cells, however, a difference in TGF-β1 mRNA levels between control and TGF-β1-treated cells can only be detected 18 h after placing cells in experimental conditions. In contrast to 3T3 cells, control NRK cells exhibit a high constitutive level of TGF-β1 mRNA expression, which persists for at least 6 h after shifting them from 5% serum (which contains TGF-β1) to serum-free medium at the beginning of the experiment.

To determine whether protein synthesis is required for positive autoregulation of TGF-β1 mRNA, we examined the effect of cycloheximide on TGF-β1 messager expression in control and TGF-β1-treated cells. Once again, we observed quite different responses in NIH 3T3 and NRK cells. In NIH 3T3 cells, blocking protein synthesis has minimal effects on TGF-β1-stimulated levels of TGF-β1 mRNA, but cycloheximide treatment significantly increases control TGF-β1 mRNA levels by 6 h of treatment. This superinduction of TGF-β1 mRNA in 3T3 cells following cycloheximide treatment implies that short half-lived negative regulatory factors

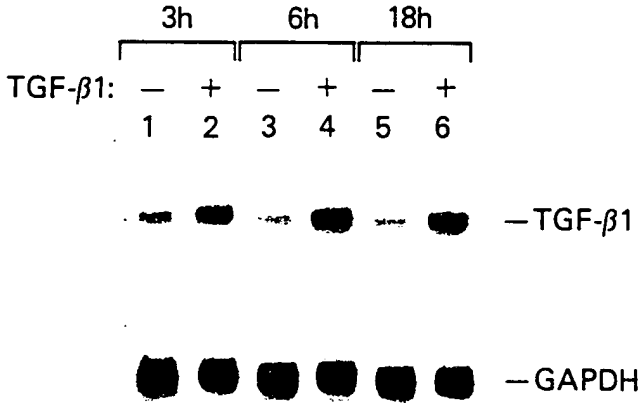

FIG. 2. **Time course of TGF-β1 effects on TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase expression in ROS cells.** Total RNA was isolated from cells following 3, 6, or 18 h of treatment in serum-free medium with no addition (−) or with 5 ng/ml TGF-β1 (+). The Northern blot (12 μg of RNA/lane) was hybridized using TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes.

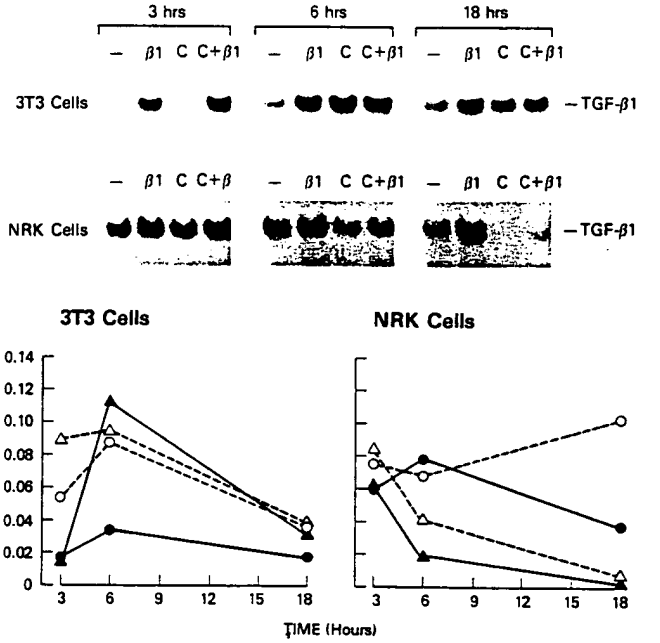

FIG. 3. **TGF-β1 expression in NIH 3T3 and NRK cells following treatment with TGF-β1 and cycloheximide.** *Upper*, Northern analysis was performed on total RNA (12 μg/lane) from NIH 3T3 and NRK cells following treatment for 3, 6, or 18 h in serum-free medium containing 5 ng/ml TGF-β1 and/or 25 μg/ml cycloheximide (*C*) as indicated (+). *Lower*, quantification of the effect was performed by densitometric scanning of the autoradiogram (*upper*); results are expressed in arbitrary units. Time course of the effect: no addition (●) and TGF-β1 (○). Effect of cycloheximide on TGF-β1 mRNA levels: cycloheximide (▲) and cycloheximide plus TGF-β1 (△).

are operant in these cells. In contrast, TGF-β1 mRNA expression is not increased by cycloheximide in NRK cells. In fact, blocking protein synthesis causes a parallel decrease in both control and TGF-β1-stimulated levels of TGF-β1 mRNA. Clearly, there are differences between these two cell lines with respect to the positive and negative regulatory elements acting at any of several levels to alter TGF-β1 mRNA expression.

Effect of TGF-β1 on Synthesis of TGF-β1 Protein—To determine whether the accumulation of TGF-β1 transcript induced by TGF-β1 reflects a corresponding increase in synthesis of the protein, the rates of TGF-β1 synthesis and secretion into the culture media of control and treated cells were compared. For these experiments, culture media from ^{35}S-labeled NRK cells following an overnight incubation in the presence or absence of TGF-β1 were immunoprecipitated with anti-TGF-β1 antibodies and analyzed by SDS-polyacrylamide gel electrophoresis. Indeed, the autoradiogram pictured in Fig. 4 demonstrates that NRK cells treated with TGF-β1 (*lane 3*) secrete 2.7-fold more TGF-β1 into their culture medium than do untreated cells (*lane 1*) over the same period of time. The cell-associated pool of [^{35}S]cysteine was essentially the same in treated *versus* untreated cultures, indicating that transport of the labeled amino acid was not a limiting step (not shown). Thus, the magnitude of the TGF-β1 effect on its mRNA levels is consistent with its effect on the secreted protein product.

Induction of TGF-β1 mRNA Expression by Purified Growth Factors—We next examined whether other purified growth factors were able to regulate expression of TGF-β1 in NRK cells. EGF, a mitogen for these cells, caused a 2.1-fold increase in TGF-β1 mRNA levels in the experiment presented in Fig. 5. More important, the combined effect of EGF and TGF-β1 on TGF-β1 mRNA expression in NRK cells was greater than

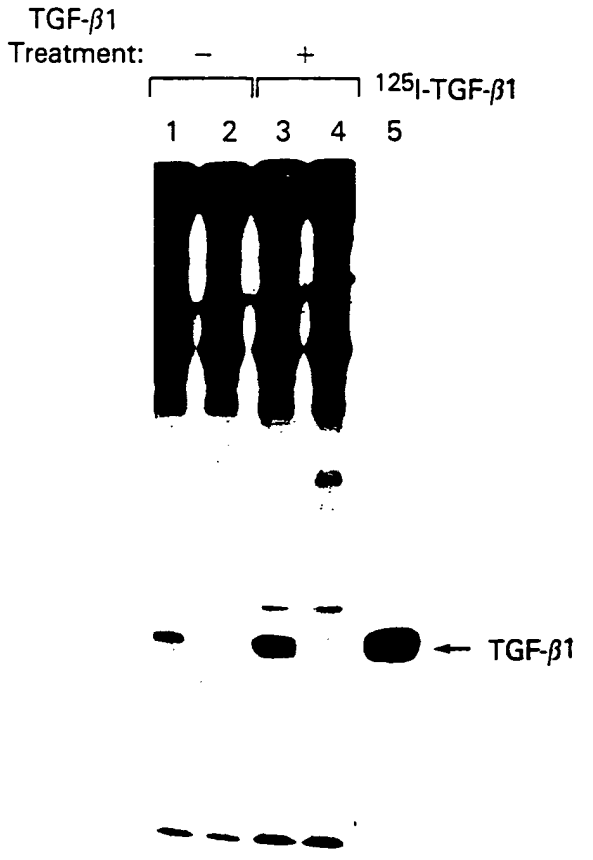

FIG. 4. **Immunoprecipitation of biosynthetically labeled TGF-β1 secreted by NRK cells.** Media conditioned by NRK cells incubated for 20 h in serum-free medium containing ^{35}S-labeled cysteine and 5 ng/ml TGF-β1 where indicated (+) were immunoprecipitated using anti-TGF-β1 antibodies. In some cases, antibodies were preincubated with excess TGF-β1 prior to immunoprecipitation (*lanes 2* and *4*). An autoradiogram of the labeled proteins following SDS-polyacrylamide gel electrophoresis is presented. ^{125}I-labeled TGF-β1 was run in *lane 5* for comparison. Densitometric scanning of the autoradiogram revealed a 2.7-fold increase in the band corresponding to TGF-β1 in treated cells after correcting for nonspecifically immunoprecipitated proteins.

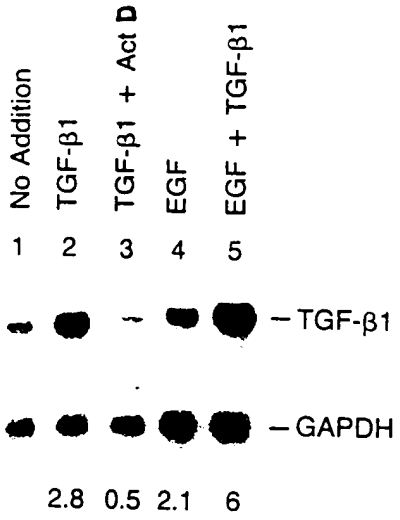

FIG. 5. **Effect of EGF and actinomycin D on TGF-β1 expression in NRK cells.** Northern blot analysis of TGF-β1 expression was performed on total RNA (12 μg/ml) from cells treated for 18 h in serum-free medium with no addition, TGF-β1 (5 ng/ml), TGF-β1 plus actinomycin D (*Act D*; 10 μg/ml), EGF (10 ng/ml), or TGF-β1 plus EGF. Autoradiograms showing TGF-β1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression on the same blot are presented; -fold stimulation of TGF-β1 expression above the untreated control level was determined by quantitative densitometric scanning. Data were not normalized to glyceraldehyde-3-phosphate dehydrogenase levels since, in three separate experiments, it has been shown that EGF induces glyceraldehyde-3-phosphate dehydrogenase expression.

TABLE I

Growth factor induction of TGF-β1 and type I collagen mRNA expression

Northern analyses were performed on total RNA from NRK cells treated for 18 h in the presence or absence of the indicated growth factor. To determine TGF-β1 and type I collagen mRNA expression levels, blots were hybridized sequentially with TGF-β1 and α2(I) collagen probes; the intensity of the autoradiographic signals was quantitated by densitometric scanning. -Fold stimulation of mRNA expression over that in untreated control cells is presented.

Effector	Stimulation	
	TGF-β1	α2(I) collagen
	-fold	
TGF-β1 (5 ng/ml)	3.8	4.15
TGF-β2 (5 ng/ml)	2.8	4.47
EGF (10 ng/ml)	3.7	1.32
PDGF (10 ng/ml)	2.5	1.13

that of either effector alone, suggesting the use of different activating mechanisms by these factors. Supporting this, recent experiments have shown that TGF-β1 mRNA levels in NRK cells treated for 24 h with EGF are unaffected by the addition of cycloheximide (data not shown), whereas the levels in cells treated in a similar fashion with TGF-β1 are severely reduced in the presence of cycloheximide (see Fig. 3). TGF-β1-induced stimulation of TGF-β1 mRNA expression appears to occur, at least in part, at a transcriptional level since actinomycin D abrogates its stimulatory effect (Fig. 5, *lane 3*). In addition to EGF, PDGF and TGF-β2, the recently characterized TGF-β1 homolog (25), elicited increases in TGF-β1 mRNA levels in NRK cells (Table I).

Coordinate Expression of TGF-β1 and Type I Collagen—TGF-β1 has been shown to stimulate synthesis of extracellular matrix components, including collagen (10, 11, 36, 37) and fibronectin (10, 37) in cultured cells. When we compared the expression of TGF-β1 and type I collagen in NRK and NIH 3T3 cells treated with TGF-β1, strikingly similar patterns were observed. As illustrated in Fig. 6 (*upper*), TGF-β1 is effective in stimulating both type I collagen and TGF-β1 mRNA expression in NRK cells over the same range of concentrations ($ED_{50} \sim 0.1$–0.3 ng/ml). Furthermore, blocking protein synthesis with cycloheximide causes a parallel effect on levels of α2(I) collagen and TGF-β1 mRNAs in control and TGF-β1-treated NRK cell (Fig. 6, *lower*). Thus, both the basal expression and the TGF-β1-stimulated expression of these transcripts appear to be coordinately modulated. These findings of parallel expression of TGF-β1 and type I collagen mRNAs can be extended to NIH 3T3 cells, although the time course of the response to TGF-β1 and the effect of cycloheximide on that response differ in these two cell types (data not shown).

Unlike TGF-β1, EGF and PDGF are unable to induce type I collagen expression in NRK cells (Table I). This illustrates

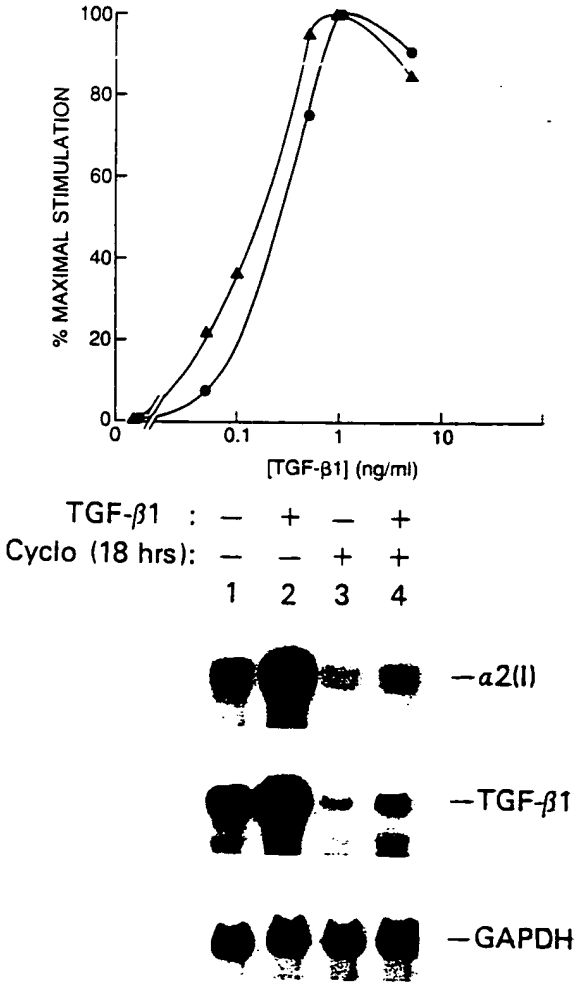

FIG. 6. **Comparison of TGF-β1, type I collagen, and glyceraldehyde-3-phosphate dehydrogenase expression in TGF-β1-treated NRK cells.** *Upper*, dose response of the TGF-β1 effect. A Northern blot of total RNA (15 μg) from NRK cells treated for 18 h with increasing concentrations of TGF-β1 in serum-free medium was hybridized with TGF-β1 (●) and α2(I) collagen (▲) probes; the resulting autoradiographic signals were quantitated by densitometric scanning. Maximal stimulation of TGF-β1 and α2(I) collagen expression in this experiment corresponds to 2.0- and 4.7-fold, respectively. *Lower*, Northern blot analysis performed on total RNA (15 μg) from cells treated for 18 h in serum-free medium with 5 ng/ml TGF-β1 and/or 25 μg/ml cycloheximide (*Cyclo*) as indicated (+). The blot was hybridized sequentially with α2(I) collagen, TGF-β1, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes.

another example of differences in the mechanisms of TGF-β1 and EGF action.

DISCUSSION

In this report, we show that TGF-β1 increases steady-state levels of its own message 2–3-fold in both normal and transformed cells. Furthermore, secretion of the corresponding protein is stimulated ~3-fold in NRK cells treated with TGF-β1. These findings add an endogenous amplification step to the autocrine model of growth factor secretion (38). Although the functional significance of this phenomenon is not known, the ability of TGF-β1 to stimulate its own production may provide a way to amplify and sustain the physiological process of wound healing, as well as carcinogenesis. Matrix production by fibroblasts is central to granulation tissue of a healing wound and to tumor stroma. As has been shown previously, TGF-β1 markedly enhances the formation of extracellular matrix by directly stimulating synthesis of major matrix proteins such as collagen (10, 11, 36, 37) and fibronectin (10, 37). Furthermore, TGF-β1 limits the destruction of extracellular matrix components by decreasing levels of secreted proteases that break down the matrix (14, 15, 19) while increasing levels of protease inhibitors (14, 15, 39). Thus, the acute release of TGF-β1 by platelets during degranulation (40) at the site of a wound or continuous secretion of TGF-β by tumor cells would trigger local synthesis of the protein by target fibroblastic cells and thereby sustain the accumulation of connective tissue matrix.

With respect to the well-documented antiproliferative effects of TGF-β1 on many cell types (35, 41, 42), release of a potent antimitogen during wound repair may represent an important negative feedback mechanism to limit cell proliferation. Previously, PDGF has been reported to induce expression of the growth inhibitor β-interferon in 3T3 cells (43). As we have observed in this study, PDGF and EGF are also capable of increasing TGF-β1 expression in NRK cells. Indeed, production of intrinsic growth inhibitors following mitogenic stimulation of cells may be essential for restraining cell growth in the presence of mitogenic factors, especially in light of recent data demonstrating that PDGF (44) and TGF-α (45) enhance both the expression of their own genes and the release of their respective protein products into culture medium of normal cells.

Our results show that increased expression of TGF-β1 mRNA is a general response to TGF-β1 in several normal and transformed cell lines; however, the site(s) at which TGF-β1 amplifies its own mRNA expression (*i.e.* transcriptional, post-transcriptional) remains to be determined. The observations that TGF-β1 and type I collagen mRNAs are coordinately modulated in NRK and NIH 3T3 cells by TGF-β may provide important clues to the mechanistic aspects of TGF-β1 gene regulation. Recent experiments (11) indicate that TGF-β1 directly activates transcription of the mouse type I collagen gene and that the effect is mediated by a nuclear factor 1-binding site located in the α2(I) collagen promoter. Our findings of parallel effects of TGF-β1 on type I collagen and TGF-β1 expression suggest that a nuclear factor 1-binding site may also be involved in autoregulation of the TGF-β1 gene. Experimental evidence in support of this hypothesis is eagerly awaited; indeed, examination of genomic sequences upstream of the human TGF-β1 precursor cDNA (46) has revealed the presence of a consensus sequence for nuclear factor 1.[2] Although the initiation site for TGF-β1 mRNA has yet to be unambiguously assigned, the nuclear factor 1 consensus element is located at position −268, relative to the most 5′-residue reported for human cDNA clones (32), and is present in reverse orientation. It is likely that regulation of TGF-β1 expression occurs at multiple levels in various cell systems. As we observed in this study, TGF-β1 and EGF appear to regulate TGF-β1 gene expression via distinct pathways. Elucidation of how TGF-β1 modulates TGF-β1 mRNA levels should provide new insights into the mechanism by which TGF-β1 itself regulates the expression of other cellular genes.

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